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The Association between Plasma 25OHD₃ Concentrations, C-Reactive Protein Levels, and Coronary Artery Atherosclerosis in Postmenopausal Monkeys

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Abstract

Objective—To identify potential relationships between plasma 25OHD, C-reactive protein (CRP), coronary artery atherosclerosis (CAA), and coronary artery remodeling in monkeys consuming atherogenic diets.

Methods—Female cynomolgus monkeys (n=74) were fed a casein-lactalbumin (C/L) based, moderately atherogenic diet for 12 months. They then consumed either a soy (n=35) or C/L (n=39) based diet for 32 months. CRP concentrations were then determined and monkeys underwent surgical menopause. Each diet group was then re-randomized to receive soy (n=36) or C/L (n=38). After 32 post-menopausal months, 25OHD, CRP, CAA, and coronary artery remodeling were determined. All monkeys received a women's equivalent of 1,000 IU/day of 25OHD₃ and 1,200 mg/day of calcium, throughout the study.

Results—The pre and post-menopausal dietary protein sources had no effect on post-menopausal 25OHD₃ concentrations (p=0.6). Across treatment groups, there was a statistically significant inverse relationship between 25OHD₃ concentrations and CRP at necropsy (r=-0.35, p=0.003). A significant inverse correlation between 25OHD₃ concentration and the change in CRP, from pre-menopause to post-menopause, was observed (r=-0.32, p=0.007). The significant associations identified between plasma 25OHD₃ and CRP remained after controlling for postmenopausal diet. Those monkeys with a greater increase in CRP also had significantly more CAA and less ability to maintain normal lumens by remodeling.

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These data were presented in abstract form September 22nd, 2011 at the NAMS 22nd annual Meeting in Washington, DC. These data and results, however, have not been published in manuscript form and have not been submitted previously to another journal.

Conflict of Interest: Thomas B. Clarkson, DVM is a member of an advisory committee to Pfizer pharmaceuticals and has been supported with a research grant from Pfizer. He is also the recipient of a research grant from Merck. The other authors have no conflicts of interest.

Conclusions—Higher plasma concentrations of 25OHD₃ were associated with lower CRP. Lower CRP was associated with less coronary atherosclerosis and improved coronary artery remodeling. These findings suggest that 25OHD₃ concentrations are associated with an anti-inflammatory state and may support an association between oral 25OHD₃ and cardioprotection.

Keywords

Plasma 25OHD₃; C - reactive protein; Cardioprotection; Coronary heart disease; Menopause; coronary artery remodeling

Introduction

It has been estimated that approximately one-third of forty year old women in the United States will develop coronary heart disease (CHD)¹. Despite recent declines, CHD remains the number one cause of mortality in woman 65 years of age^{2,3}. Furthermore, approximately 64% of women who die of CHD had no prior symptoms³. Primary risk factors for CHD in women, as determined by the National Cholesterol Education Program (NCEP), the American Heart Association (AHA), and the American College of Cardiology (ACC), include a personal history of CHD, age over 55, dyslipidemia, family history of premature CHD, diabetes mellitus, smoking, hypertension, obesity, physical inactivity, the metabolic syndrome, and a personal history of peripheral arterial disease²⁻⁵. However, the aforementioned risk factors may still underestimate risk in early postmenopausal women. Among young (~55 years of age), non-diabetic postmenopausal women classified as low-risk and requiring no further intervention according to their Framingham risk score, approximately 33% have detectable coronary artery calcium indicative of subclinical atherosclerosis⁶. Consequently, additional markers have been evaluated to determine their usefulness as indicators of CHD risk. For example, the inflammatory risk marker C-reactive protein (CRP) has been found to be an independent predictor of future cardiovascular events⁷.

In recent years, plasma 25OHD has become a topic of major interest in the research community. Although the relationship between 25OHD and bone health is well studied⁸, the current understanding of 25OHD and its direct effect on other physiologic systems is limited. Accordingly, the Institute of Medicine recently reaffirmed the need to advance our knowledge related to 25OHD deficiency and its association with the non-skeletal parameters⁸. Additionally, it has been well established that inflammation plays an important role in the pathogenesis of cardiovascular disease^{7,9}. The assessment of inflammatory markers, therefore, has been proposed as an approach to improve risk prediction of cardiovascular events. Among the biomarkers of inflammation, CRP appears to have the strongest independent ability to predict cardiovascular events⁹. More than a dozen prospective epidemiological studies have demonstrated that a single, non-fasting measure of CRP is a strong predictor of future cardiovascular events among individuals without a prior history of cardiovascular disease⁷.

Recent studies have suggested there may be a relationship between plasma 25OHD concentrations and CHD risk^{10,11}. A higher incidence of CHD and hypertension in higher latitudes, with less sunlight, has been identified in the results of previous studies. For instance, those with a lower exposure to ultraviolet light, and hence a lower vitamin D concentration, may have a higher risk of heart disease, CHD, myocardial infarction, and hypertension¹²⁻¹⁵. An association between 25OHD and CHD, therefore, could be particularly relevant to elderly women who are at risk for 25OHD deficiency due to an age-associated decline in skin photoisomerization of 7-dehydrocholesterol¹⁶ and decreased dietary intake of 25OHD₃. Additional risk factors for 25OHD deficiency include, living

further from the equator, impaired hydroxylation, impaired renal function, and end organ insensitivity¹⁷⁻²².

The majority of studies investigating the association between plasma 25OHD concentrations and CHD in human subjects are retrospective observational studies. The study presented here utilizes a cross sectional study design of cynomolgus monkeys in which oral 25OHD₃ intake, sun exposure, and activity level are controlled, while atherosclerosis extent and arterial remodeling are measured directly at the tissue level. The objective of this study, therefore, was to determine the relationships between 25OHD₃, CRP, coronary artery atherosclerosis, and coronary artery remodeling.

Methods

Animals and Diets

Adult female cynomolgus monkeys (*Macaca fascicularis*; n = 74) were used for this study. The monkeys were imported from Indonesia (Institute Pertanian Bogor), adult status was confirmed, and ages were estimated by epiphyseal closure and dentition. Upon arrival, monkeys underwent a 12 month pre-experimental period, during which they received a controlled casein-lactalbumin (C/L) atherogenic diet. After this period, monkeys were randomized to a diet containing as its main protein source either soy or C/L. Both diets contained identical amounts of fat, carbohydrate and protein per calorie of diet. The diets were equivalent in 25OHD₃ and calcium (women's equivalent 1,000 IU/day and 1,200 mg, respectively). After consuming the diet for an additional 32 months, the monkeys underwent ovariectomy to induce surgical menopause. At this time the monkeys were re-randomized to soy or C/L such that the pre and post menopausal dietary combinations included soy-soy, soy-C/L, C/L-soy, or C/L-C/L. Monkeys consumed the diets for an additional 32 months post-ovariectomy. Body composition measurements were obtained at ovariectomy and at necropsy. An adiposity index for the monkeys was derived by measuring body weight by trunk length, and has a normal range of 35 to 55 kg/m². Trunk length is measured from the sternal notch to the pubic symphysis.

All procedures involving animals in this study were conducted in compliance with the state and federal laws, standards of the US Department of Health and Human Services (DHHD), and guidelines established by the Wake Forest University Institutional Animal Care and Use Committee (IAcuC) where the animals were housed.

C-reactive Protein Measurement

CRP was measured prior to and 32 months post ovariectomy. Serum CRP was determined by ELISA using a human-based assay from ALPCO Diagnostics (Salem NH). Intra-assay and interassay coefficients of variation were less than 10%.

25OHD₃ Measurement

Plasma 25OHD₃ concentrations were measured in all 74 female cynomolgus monkeys 32 months post ovariectomy. All animals were housed indoors during the entire length of the study, minimizing any effect of sun exposure. All 25OHD assays were done at The Reading Hospital and Medical Center. Frozen samples (500 ml at -70°C) were transported to The Reading Hospital and Medical Center and protected from direct sunlight as per previously recommended techniques for yielding stable results²³. The samples had never been thawed prior to the 25OHD assessments and HPLC - tandem mass spectrometry was used for the 25OHD assays (both 25OHD₂ and 25OHD₃). Our approach utilized the Shimadzu liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/2) technology. The liquid chromatography-mass spectrometry prepares the sample to be ionized, through physical

separation capabilities of liquid chromatography, for mass analysis by injection into the AB Siex 3200 Q Trap mass spectrometer. Vitamin D was provided in the diet as 25OHD₃, so all analyses were done with plasma concentrations of 25OHD₃.

Coronary Artery Atherosclerosis

At necropsy, the coronary arteries were perfusion fixed with 10% neutral buffered formalin at a pressure of 100 mm Hg. The left anterior descending (LAD), left circumflex (LCX), and right (RCA) coronary arteries were evaluated histomorphometrically to determine both plaque thickness (maximal intimal thickness [MXIT]) and plaque area (intimal area [IA]) as described previously^{24,25}. The mean MXIT and IA for each of the three coronary arteries was then determined and the outcome variable for each was reported and analyzed, independently, as coronary artery atherosclerosis.

Coronary Artery Remodeling

After determining the degree of coronary artery atherosclerosis, as described above, the degree of coronary artery remodeling was assessed. The remodeling process, in which luminal area increases as arterial plaque size increases, has been described previously for both human subjects and macaques²⁴. In this study, remodeling was assessed by comparing the coronary artery plaque size to the luminal area. The degree of coronary artery remodeling was assessed by comparing the plaque size to artery size ratio, plaque size to luminal area ratio, and luminal area to artery size in the three coronary arteries.

Relationships among 25OHD₃, CRP and Atherosclerosis

Relationships between CRP changes and coronary artery atherosclerosis among all three coronary arteries were determined. The relationships between 25OHD₃ and CRP at necropsy, as well as the relationship between 25OHD₃ and the percent change, and absolute change, in CRP during the postmenopausal phase, were investigated. We analyzed those with high (≥ the mean) and low (< the mean) plasma concentrations of 25OHD₃ along with high (≥ the mean) and low (< the mean) plasma CRP concentrations. Using these parameters, we compared those with high 25OHD₃ / low CRP, high 25OHD₃ / high CRP, low 25OHD₃ / low CRP, and low 25OHD₃ / high CRP. Also, hypothesizing that those with high 25OHD₃ / low CRP would have the lowest degree of coronary artery atherosclerosis, we studied this group compared with all the others combined.

Statistical analysis

Body weight data are presented as means and standard deviations. 25OHD₃ concentrations were assessed for normality of distribution and were treated as continuous variables. Subjects were dichotomized into high and low 25OHD₃ concentration groups based on the determined mean concentration of 25OHD₃. Each parameter of interest was compared between two 25OHD₃ concentration groups either by student's t-test or Mann-Whitney test depending upon whether or not data was normally distributed. For assessment of correlation between demographic variables and 25OHD₃ concentrations of the entire cohort (n=74), Pearson's correlation was used for normally distributed continuous variables, and Spearman's ranked correlation for non-normally distributed variables. Correlations between CRP and 25OHD₃ concentrations (considered as both categorical [high vs. low] and continuous variables) were analyzed using Spearman's ranked correlation. Multiple linear regression analysis was employed to assess predictive relationships between 25OHD₃ concentrations and each parameter of interest. All comparisons were done at both the end of menopause (at necropsy) and for the postmenopausal period (ovariectomy to necropsy). SAS 9.2 was used for all analyses, a value of p<0.05 was used to determine a statistical significance.

During construction of figure 3, comparing CRP and coronary artery atherosclerosis as a continuous variable, there was one atherosclerosis data point that was noted to be an outlier value for the intimal area parameters. The data point was more than 10 times higher than the other values. While this value was determined to be a true value, the inclusion in the figure made the graphic representation difficult. While the value was included in the results and data analysis, this subject was excluded from figure 3 in order to eliminate a disproportionate scale. Examination of analytic results was performed, however, and the p value remained unchanged after elimination of this outlier value.

Results

Of the 74 monkey's, thirty nine consumed the C/L diet and thirty five consumed the Soy diet during the premenopausal phase (baseline to ovariectomy). During the postmenopausal phase (ovariectomy to necropsy), thirty eight consumed the C/L diet and thirty six consumed the Soy diet. At the end of the postmenopausal life phase, plasma 25OHD₃ concentrations ranged from 1.4 to 102 with a mean of 48.9 +/- 16.6 ng/mL. The estimated mean age was 21.5 +/- 3.2 years, the mean weight was 3.4 +/- 0.7 kg and the mean adiposity index was 47.6 +/- 8.7 kg/m². See table 1 for additional demographic information.

After being postmenopausal for 32 months, monkeys with a higher plasma 25OHD₃ concentration (i.e. the mean) had significantly lower CRP concentrations (750.6 ng/ml +/- 698.1 vs. 1042.3 ng/ml +/- 994.4, respectively, $p = 0.04$; Effect size Cohen's $d = -0.36$; figure 1) than the group with low 25OHD₃. Similarly, the continuous data also show a significant inverse relationship between plasma 25OHD₃ and serum CRP at necropsy ($p = 0.003$, $r = -0.35$), figure 2A. When controlling for the postmenopausal diet (soy versus C/L) in a multiple linear regression analysis, this relationship was unchanged ($p = 0.0004$, Adjusted R-Square = 0.15). The relationship between the percent change in CRP from the end of the premenopausal period until the end of the postmenopausal time period (32 months) and 25OHD₃ concentrations was also inverse ($r = -0.32$, $p = 0.006$), figure 2B. When controlling for the postmenopausal diet (soy vs. C/L) in a multiple linear regression analysis, the relationship was again unchanged ($p < 0.0001$, Adjusted R-Square = 0.20). Similarly, as the plasma concentration of 25OHD₃ increased, the absolute increase in CRP was significantly lower ($p = 0.007$, $r = -0.31$). Again, when we controlled for the postmenopausal diet (soy vs. C/L) in a multiple linear regression analysis, this relationship was still significant ($p = 0.0006$, Adjusted R-Square = 0.19).

As the absolute CRP increased, atherosclerotic plaque area (IA) and maximal intimal thickness (MXIT) were increased in all three main coronary arteries (LAD, LCX, and RCA), Figure 3. LAD atherosclerosis extent was decreased in monkeys with high plasma 25OHD₃ concentrations (in the upper half of the mean) and low CRP change (in the lower half of the mean) compared to all other monkeys (those without high 25OHD₃ and low CRP change), $p = 0.03$, Cohen's $d = 0.50$, figure 4. This relationship was also observed with plaque size (MXIT) in the RCA ($p = 0.04$, Cohen's $d = 0.58$), but not in the LAD or LCX.

We also observed the usual association between the ratio of mean lumen area to mean intimal area (plaque size) of the LCX, LAD, and RCA. As coronary artery plaque size (IA) increased there was a significant association with increases in the lumen area (LA) of all three main coronary arteries (LCX, $r = 0.57$, $p < 0.0001$; LAD, $r = 0.55$, $p < 0.0001$; RCA, $r = 0.25$, $p = 0.0355$), indicative of artery remodeling. However, remodeling appeared to be compromised in monkeys that experienced an increase in CRP concentration during the menopausal years. CRP was positively associated with the intimal area:artery size ratios of the LCX, LAD, and RCA. This finding represents a consistent finding, in all three main coronary arteries, of a correlation between a greater absolute increase in CRP during the

menopausal years and a significant compromise in coronary artery remodeling (LCX, $r = 0.32$, $p=0.007$; LAD, $r = 0.32$, $p=0.007$; RCA, $r = 0.33$, $p=0.005$). Finally, we showed that the absolute change in CRP was inversely related to the luminal area:artery size ratio for all three main coronary arteries (LAD, LCX, and RCA). This again confirmed an enhanced coronary artery remodeling with a lower CRP increase during menopause (figure 5).

Discussion

The results of our study demonstrated an inverse relationship between plasma concentrations of 25OHD₃ and CRP. The association was apparent when considering 25OHD₃ both as a continuous variable and when comparing higher versus lower 25OHD₃ plasma concentrations. We also demonstrated that higher CRP concentrations, as well as increasing CRP concentrations during the menopause, were associated with greater coronary atherosclerosis.

What does the relationship between 25OHD₃ and CRP identified in our study mean? Certainly CRP is an acute phase reactant and as such has been shown to rise dramatically during inflammatory processes occurring in the human body^{26,27}. Current research has demonstrated that these markers play significant roles in a variety of acute and chronic diseases including infections, systemic inflammatory processes, tissue injury, hypertension, the metabolic syndrome, and most notably CHD^{26,27}. 25OHD₃ has been recognized as an immune-modulator that may suppress pro-inflammatory cytokine expression and regulate immune cell activity^{28,29}. Additionally, it is believed that 25OHD₃ supplementation may be associated with a reduction in pro-inflammatory cytokines in patients with heart failure³⁰. It is important to note that this study was not designed to assess the effects of differential Vitamin D supplementation, as all animals received equivalent levels of 25OHD₃ in their diet. Individual variation in circulating 25OHD₃ is likely due, in part, to genetic determinants and responses to atherogenic diets, which may influence absorption and degradation in the liver, kidney, and elsewhere. Since CRP is produced by the liver, it is possible the inverse relationship between 25OHD₃ and CRP is related to hepatic function, and that hepatic inflammation causes increased 25OHD₃ turnover. More work needs to be done to explore these potential mechanisms. While there is some evidence to suggest 25OHD deficiency may be associated with higher rates of cardiovascular disease^{10,11,31}, well designed prospective data to support this belief are lacking⁸. Additionally, at least one recent randomized study did not show that 25OHD₃ supplementation changed circulating cytokine levels among healthy adults³².

Some of the best data available, in fact, has also not been consistent with a cardio-protective role in regard to 25OHD. For example, data from the Women's Health Initiative, analyzing 36,282 postmenopausal women randomized to calcium and 25OHD₃ supplementation, was recently published³³. This study suggested that calcium and 25OHD₃ supplementation was not associated with a risk of coronary or cerebrovascular events in generally healthy postmenopausal women over a 7-year period. Due to limitations of the study, however, it is still premature to draw definitive conclusions based on these findings. While these data are from a prospective trial, it was originally designed to evaluate the effects of calcium and 25OHD₃ supplementation on fracture, not cardiovascular disease. Also, the dose of 25OHD₃ (400 IU daily) might be inadequate and insufficient to achieve optimal plasma 25OHD₃ concentrations^{33,34}. In addition, the proportion of patients with coexisting hypertension at baseline was significantly higher in the 25OHD₃ supplementation group. Since clinical evidence of 25OHD as a protective factor for cardiovascular disease in postmenopausal women is limited, additional data on potential mechanisms for cardioprotection are necessary. The inverse relationship between plasma 25OHD and CRP observed here suggests either an anti-inflammatory, and perhaps cardioprotective, effect of Vitamin D or a

potential explanation, i.e. systemic inflammation, for a low 25OHD₃ concentration in those with cardiovascular disease.

While a direct causal relationship between elevated CRP and coronary artery atherosclerosis in human subjects has not been proven, CRP is nevertheless frequently used in CHD risk assessment algorithms^{9,35-37}. Not shown previously, for either human or nonhuman primates, is an association between coronary artery remodeling and plasma CRP concentrations. Despite the difficulty proving this in human cohorts, we were able to document that the increase in CRP over time, and during the menopausal years, correlated with extent of coronary artery atherosclerosis. We found that those with the highest plasma 25OHD₃ concentrations and the lowest increase of CRP over time, as expected, were afforded the greatest protection against coronary artery atherosclerosis. We also determined that those with the smallest increases in CRP during the menopausal period had the greatest degree of coronary artery remodeling, a finding consistent with decreased risk for CHD and cardiovascular events²⁴. Clearly we have shown strong evidence that lower plasma concentrations of 25OHD₃ are associated with higher systemic inflammation, as measured by CRP, and this in turn is associated with greater coronary artery atherosclerosis and worsening coronary artery remodeling. 25OHD₃, therefore, could play a cardio-protective role evidenced by the reduction of this marker, or may at least indicate a less atherogenic milieu. In addition to identifying and correlating a direct association between 25OHD₃ and coronary artery atherosclerosis, the correlation with other disease entities, as listed above, could also prove beneficial through future prospective trials.

The limitations to this study include the following. While this cynomolgus monkey model has proven to be effective in studying menopause related chronic diseases, particularly cardiovascular disease³⁸, the relationships identified in this study may not be the same as those in human beings, hence further studies in human cohorts are needed. The small sample size in our monkey cohort may have contributed to a type 2 error, limiting the identification of further significant relationships. While it is possible that the various dietary regimens could have affected the inflammatory markers, we demonstrated that the source of dietary protein had no effect on the plasma concentrations of 25OHD₃. In addition, regression analyses controlling for the diets revealed that the dietary changes did not alter the significant associations between 25OHD₃ and CRP. Because we do not definitively know the change in 25OHD₃ concentration over the course of the study, we cannot conclusively determine whether inflammation leads to a reduced 25OHD₃ or whether a higher 25OHD₃ leads to reduced inflammation. We believe it is highly unlikely, however, that the plasma 25OHD₃ concentrations in these monkeys varied significantly over time because of the strict control over their dietary 25OHD₃ and their environment, both of which remained constant during the experiment. In addition, because genetic factors are likely responsible for at least some of the observed variations in 25OHD₃ concentrations, it is plausible that these same factors may be influencing CRP concentrations. Therefore, despite our data showing a clear association between 25OHD₃ and CRP, further prospective data will be needed to clarify this relationship along with the temporal association.

Advantages of the non-human primate model include the ability to control for many of the confounding variables in human studies, including diet, 25OHD₃ intake, sun exposure, and general compliance. Additional strengths include the elimination of various confounding factors existing in human studies such as coexisting medical conditions (i.e. type II diabetes) which has been linked to elevations in pro-inflammatory markers, especially CRP²⁸ and concurrent hormonal therapy which has been associated with increased CRP concentrations³⁹. Other confounding factors include exogenous 25OHD₃/calcium or other supplementation (known or unknown) along with exercise, which has been associated with decreased inflammatory markers including CRP⁴⁰. For these reasons, observed findings in

this model may yield results that are less biased than those in human cohorts. It will be beneficial, therefore, to examine these findings in the general postmenopausal population.

Conclusion

We have identified a significant inverse correlation between 25OHD₃ and the inflammatory marker CRP. This increase in CRP, likewise, was correlated with a greater degree of coronary artery atherosclerosis and inhibited coronary artery remodeling. This association was strong and independent of any of the menopausal dietary changes. These findings suggest that circulating 25OHD₃ concentrations are associated with an anti-inflammatory state. These findings support previous suggestions that higher 25OHD₃ concentrations may result in cardiovascular protection. Further investigations are required to determine the mechanism behind these relationships.

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Figure 1.

C-reactive protein (ng/mL) values in postmenopausal monkeys with lower vs. higher plasma 25OHD₃ concentrations (< 48.9 ng/mL vs. ≥ 48.9 ng/mL, respectively).

Figure 2.

Relationship between 25OHD₃ concentration and C-reactive protein (CRP) concentration (ng/mL) at necropsy (A) and with percent change in C-reactive protein (CRP) concentration (ng/mL) from ovariectomy to necropsy (32 months) (B) in cynomolgus monkeys (*Macaca fascicularis*).

Figure 3.

A correlation between the plaque area of the LAD and the absolute increase in CRP during the 32 month postmenopausal period is shown. A direct correlation between a greater absolute increase in CRP during the menopausal years and significantly greater amounts of coronary artery atherosclerosis, as measured by coronary artery plaque intimal area, was observed (LAD, $r = 0.29$, $p=0.015$). LAD = left anterior descending coronary artery; CRP = C-reactive protein.

Figure 4.

Left anterior descending (LAD) coronary artery atherosclerosis (mm²), in postmenopausal monkeys with high 25OHD₃ / low C-reactive protein change (ng/mL) compared to all the others. CRP change = Absolute change in C-reactive protein concentration during the menopause years; CAA = coronary artery atherosclerosis.

Figure 5.

This figure demonstrates a correlation between the luminal area:artery size ratio of the LAD vs. the absolute increase in CRP during the menopausal years. This represents a direct correlation between a greater absolute increase in CRP during the menopausal years and a significantly greater decrease in luminal area: artery size ratio, representing a compromise in coronary artery remodeling (LAD, $r = -0.32$, $p=0.007$). LAD = left anterior descending coronary artery; ASize = Artery size; LA = Luminal area; CRP = C-reactive protein.

Table 1**Demographics of four Dietary Groups**

Diet	Soy-Soy (n = 17)	Soy-C/L (n = 18)	C/L-Soy (n = 19)	C/L-C/L (n = 20)	Total (n=74)	P value*
Variables (Mean +/- SD)						
Age	21.4+/-3.0	21.3+/-3.6	21.8+/-3.1	21.3+/-3.1	21.5 +/- 3.2	0.961
Body weight (at ovariectomy) (kg)	3.4 +/- 0.7	3.5+/- 0.9	3.4+/- 0.4	3.4+/- 0.7	3.4 +/- 0.7	0.929
AI (at ovariectomy)	45.7+/-9.2	46.5+/-10.6	49.4+/-6.5	48.3+/-8.6	47.6 +/- 8.7	0.568
25OHD ₃ Concentration (ng/mL)	49.4+/-12.1	44.4+/-14.0	50.2+/-16.4	51.5+/- 22.2	48.9+/-16.6	0.599

C/L = casein-lactalbumin

SD = standard deviation

AI = Adiposity Index

* Baseline comparison of each parameter of interest between four diet groups was conducted by One-way Analysis of Variance (ANOVA)